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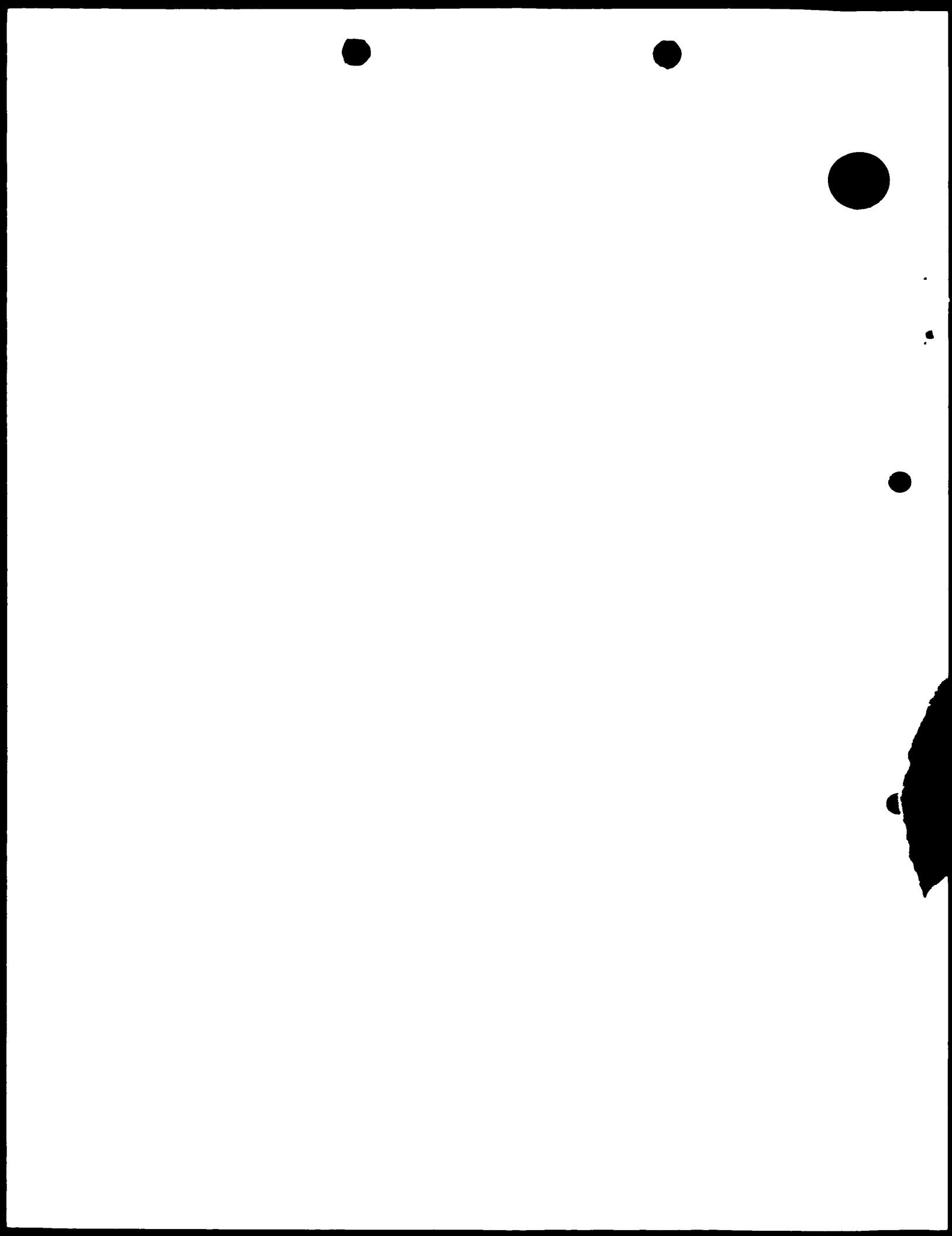
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Dated 20 January 2000

## PRIORITY DOCUMENT

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16JAN99 E418432-17 D02690  
LP01/7700 0.00 - 9900930.0

## Request for the grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

15 JAN 1999

The Patent Office

Cardiff Road  
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Gwent NP9 1RH

1. Your reference

REP05909GB

2. Patent application number

(The Patent Office will fill in this part)

9900930.0

3. Full name, address and postcode of the or of each applicant (underline all surnames)

University of Nottingham  
 University Park  
 Nottingham  
 NG7 2RD  
 United Kingdom

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

798405001

4. Title of the invention

PRO-APOPTOTIC AGENTS

5. Name of your agent (if you have one)

GILL JENNINGS &amp; EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Broadgate House  
 7 Eldon Street  
 London  
 EC2M 7LH

Patents ADP number (if you know it)

745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

YES

- a) any applicant named in part 3 is not an inventor
- b) there is an inventor who is not named as an applicant, or
- c) any named applicant is a corporate body.

See note (d))

tents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.  
Do not count copies of the same document

Continuation sheets of this form

Description 5 ✓

Claim(s) 1 ✓

Abstract

Drawing(s) 4 + 4 - 1 ✓

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination  
(*Patents Form 10/77*)

Any other documents  
(please specify)

11. For the Applicant I/We request the grant of a patent on the basis of this application.  
Gill Jennings & Every

Signature

Date

15 January 19

12. Name and daytime telephone number of person to contact in the United Kingdom PERRY, Robert Edward  
0171 377 1377

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## PRO-APOPTOTIC AGENTS

Field of the Invention

This invention relates to pro-apoptotic agents isolatable from *Necator americanus*.

5    Background to the Invention

Human nematodes (roundworms) include the hookworm nematode species, *Necator americanus*. Adult females of *N. americanus* are typically 9-11 mm in length and adult males are typically 7-9 mm in length. These adult worms commonly 10 reside in the lumen of the small intestine, and attach to the intestinal wall resulting in blood loss from the host. Eggs are passed out in the faeces and, under favourable conditions, usually hatch in 1-2 days. Larvae are then released and continue to grow in the faeces and/or the 15 soil. After up to 10 days, the larvae are infectious, and may survive 3-4 weeks in this condition. If, during this time, contact is made with a human host, the larvae can penetrate the skin, after which they may be carried through the veins and the heart to the lungs. Here, they penetrate 20 the pulmonary alveolae and ascend the bronchial tree to the pharynx where they can be swallowed and delivered to the small intestine. They then develop into adult worms. Typically, six weeks or more is required from the initial 25 infection to oviposition by the adult female.

*N. americanus* is found in tropical and sub-tropical localities, where it gives rise to a hookworm disease having a number of clinical features. Iron deficiency anaemia, resulting from blood loss at the site of 30 intestinal attachment of the adult worms, is the most common symptom of hookworm infection, and may be accompanied by cardiac complications. Gastrointestinal and nutritional/metabolic symptoms may also be found. Additionally, itching may occur during the initial 35 infection, and respiratory symptoms may be observed during the pulmonary migration stage.

Apoptosis is a suicide process built into all mammalian cells in which a cell dies in a controlled

manner. Cells undergoing apoptosis show distinctive morphological changes, for instance nuclear condensation and the formation of apoptotic bodies. The biochemical hallmark of apoptosis is the cleavage of chromatin into 5 nucleosomal fragments.

Summary of the invention

The present invention is based on the realisation that hookworms shield against immunological attack by producing a factor capable of reducing the viability of reactive T 10 cells. This factor may therefore exert an effect that results in cell apoptosis and may have valuable therapeutic application.

The present invention therefore provides a substantially pure excretory-secretory (ES) product, 15 isolatable from *N. americanus*, and functional derivatives thereof, capable of reducing cell viability. Cell viability may be reduced via the induction of apoptosis.

The invention further provides a use for these ES products and derivatives, in the manufacture of a pro-apoptotic composition. 20

The invention further provides a pro-apoptotic composition comprising a pharmaceutically-acceptable diluent or carrier, and one or more ES product or derivative.

The invention further provides ES products or derivatives for use in the manufacture of a medicament with anti-tumour and/or anti-inflammatory activity. 25

Brief Description of the Drawings

In the drawings:

Figure 1 shows the effect of *N. americanus* excretory-secretory products on the cell viability of human leukaemic T-cell line Jurkat; 30

Figure 2 shows the induction of DNA fragmentation, a hallmark of apoptosis in the human leukaemic T-cell line Jurkat by the excretory/secretory products from *N. americanus*; 35

Figure 3 shows the morphological changes typical of apoptosis in the human leukaemic T-cell line Jurkat by the excretory/secretory products from *N. americanus*; and

5 Figure 4 shows the effect of partially purified excretory-secretory products on the cell viability of the human leukaemic T-cell line Jurkat.

Description of the Invention

By way of example only, excretory-secretory (ES) products of *N. americanus* may be prepared in the following 10 manner.

*Necator americanus* is passaged in DSN hamsters. Faecal culture from the infected animals provide infective larvae, which are then used to infect neonates percutaneously. Adult worms are routinely harvested from the 15 small intestine of infected hamsters 5 weeks post-infection. The ileum of the infected hamster is removed, opened longitudinally, and placed in Hanks' saline at 37°C. As worms release their hold on the mucosa, they are carefully removed, thoroughly washed, and cleansed in 20 Hanks' saline containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Cleansed worms are examined under a dissecting microscope, and undamaged worms retained.

Under sterile conditions, worms are added to RPMI 1640, containing penicillin and streptomycin, as above. 25 The worms are then cultured for 16 hours, and the supernatants removed for analysis of pro-apoptotic activities.

Cultured supernatants are sterile-filtered through 30 0.2 µm filters, which also removes eggs that may have deposited during the culture period.

Protein concentration of the supernatants is assayed using Coomasie Brilliant Blue with BSA as standards.

To assess the effects of hookworm ES on the viability 35 of Jurkat cells,  $2 \times 10^5$  cells were cultured with various concentration of ES products in a final volume of 200 µl in flat-bottomed 96-well plates for 16 hours at 37°C in a 5% CO<sub>2</sub> incubator. This was followed by the addition of 20 µl

of Thiazol blue solution (5 mg/ml) to the cells and the plates were incubated for a further 4 hours. After the incubation, 150  $\mu$ l of medium was removed carefully from the wells, followed by the addition of 150  $\mu$ l iso-propanol, and  
5 mixed thoroughly. The OD at 590 and 650 nm was determined on an ELISA reader. Cell viability was expressed as the percentage of control absorbance obtained in untreated cells after subtracting the absorbance from appropriate blanks.

10 The induction of apoptosis in Jurkat T-cells by ES products was monitored by staining fixed cells with Hoechst dye 33358 (50  $\mu$ g/ml in PBS) and examining the nuclear morphological changes using confocal laser microscopy, and the analysis of oligonucleosomal DNA fragments in the  
15 Jurkat cells using agarose gel electrophoresis.

Figure 1 shows the effect of *Necator americanus* ES products on Jurkat cell viability. Cell viability was reduced (ie cells were killed) in a dose-dependent manner. Cell viability was shown to be reduced via the induction of apoptosis. The characteristic cleavage of chromatin into nucleosomal fragments, that is indicative of apoptosis, is demonstrated in Figure 2, an agarose gel showing the dose-dependent induction of DNA fragmentation by ES products. A further characteristic of apoptosis is the change in nuclear morphology and this was observed in the cells after treatment with ES products (Figure 3).

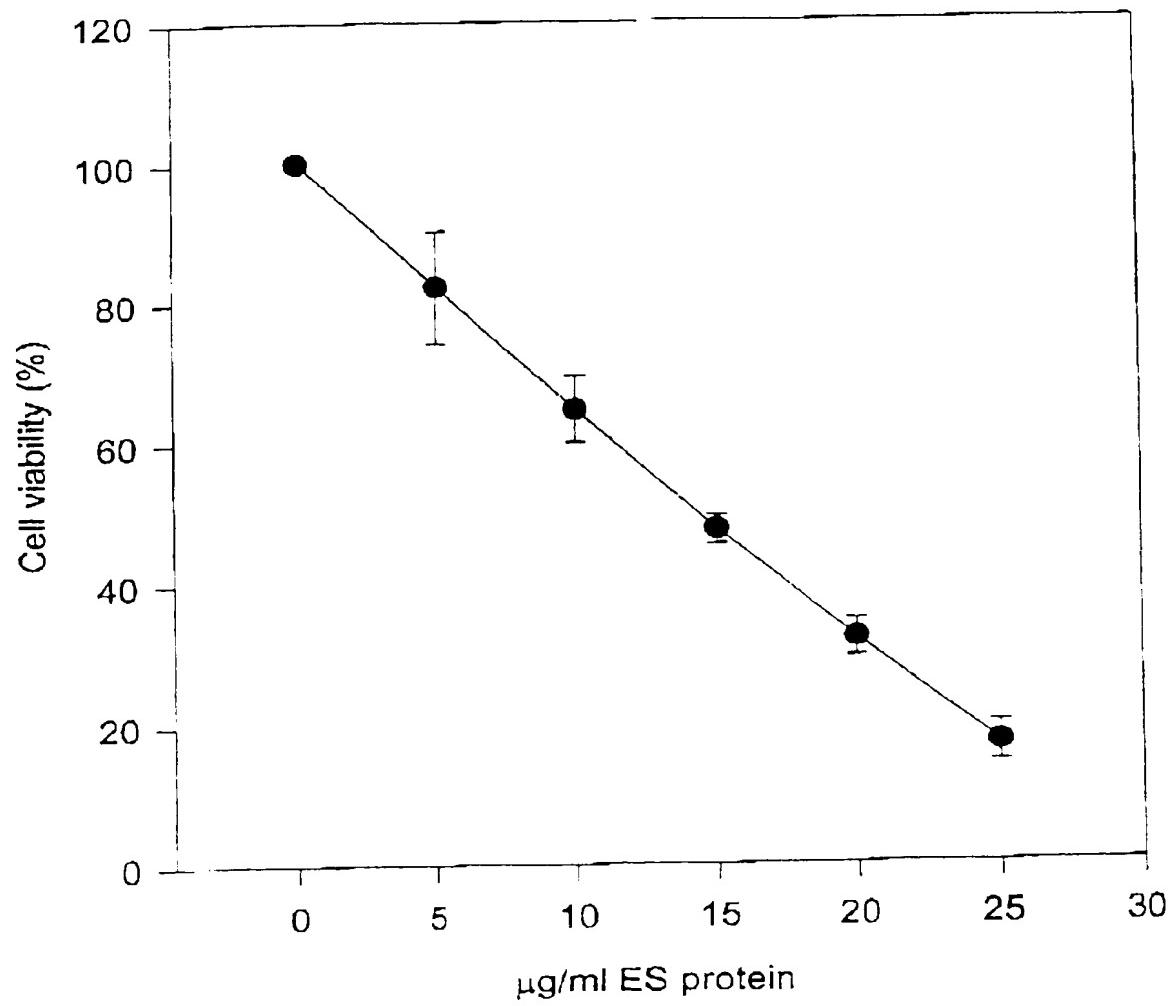
After fractionation through a Sephadryl S-300 column, the fractionated *N. americanus* preparation was assessed for pro-apoptotic activity. Each fraction was then co-cultured  
30 with Jurkat cells, and the cell viability index determined. Values of less than 1.0 indicate apoptotic cells. Figure 4 shows the cell viability index of fractions 1 to 45. Fractions 27-33 were found to have significantly lower cell viability indexes (<1.9) and therefore cell killing  
35 activities. Subsequent incubation of Jurkat cells with these fractions induced apoptosis in the cells. Fractions 27-33 were concentrated and separated on a 15% SDS PAGE.

The gel showed very little protein bands but indicated that the pro-apoptotic agent may be less than 12 kDa in size.

CLAIMS

1. A substantially pure excretory-secretory product, isolatable from *Necator americanus*, or a fragment thereof, capable of inducing apoptosis in reactive T-cells.
- 5 2. A product according to claim 1, for use in therapy.
3. A pro-apoptotic composition comprising a pharmaceutically acceptable diluent or carrier, and a product as defined in either preceding claim.
4. The use of a product as defined in any preceding  
10 claim, in the manufacture of a medicament with anti-tumour activity.
5. The use of a product as defined in any of claims 1 to 3, in the manufacture of a medicament with anti-inflammatory activity.

*Figure 1*



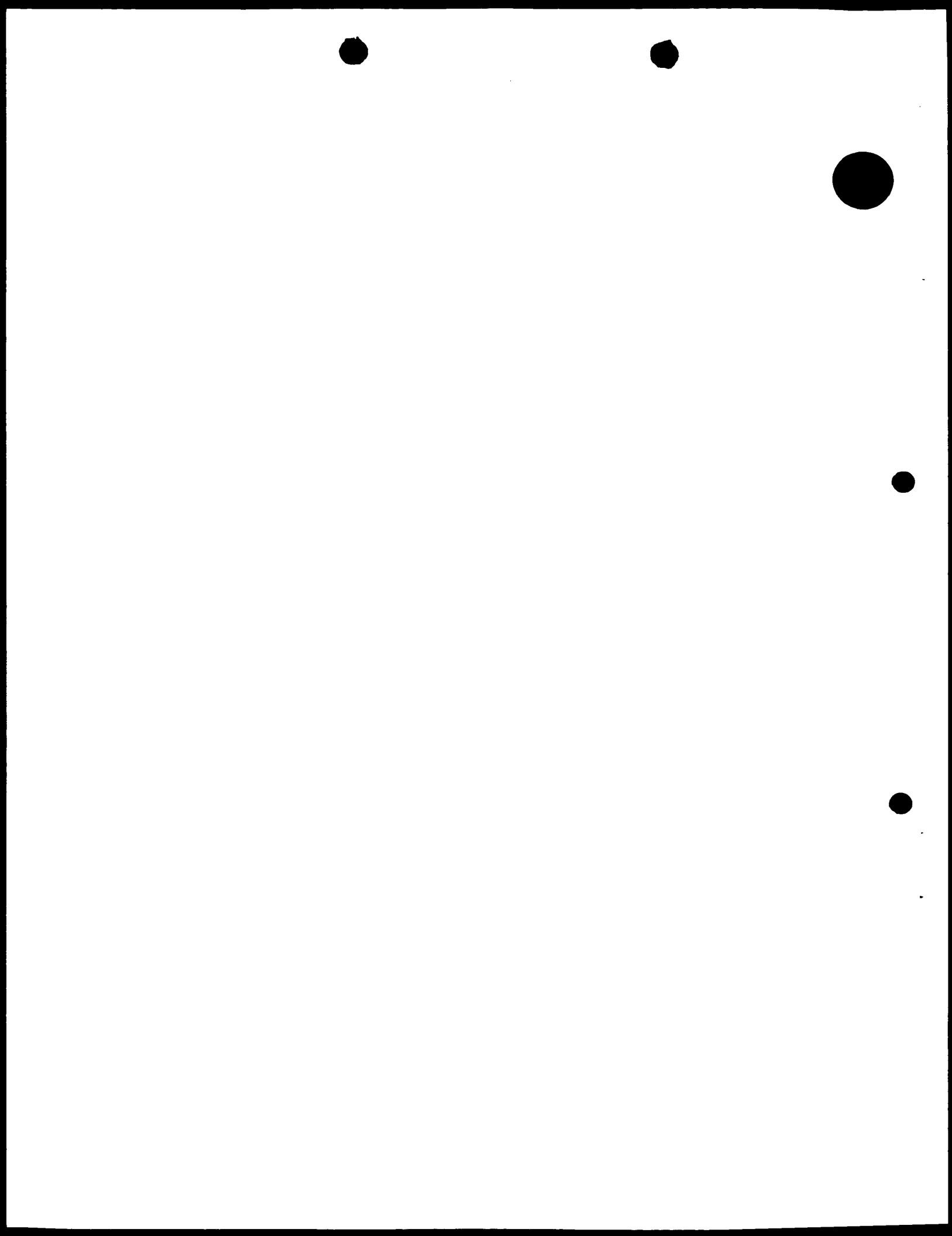


Figure 2



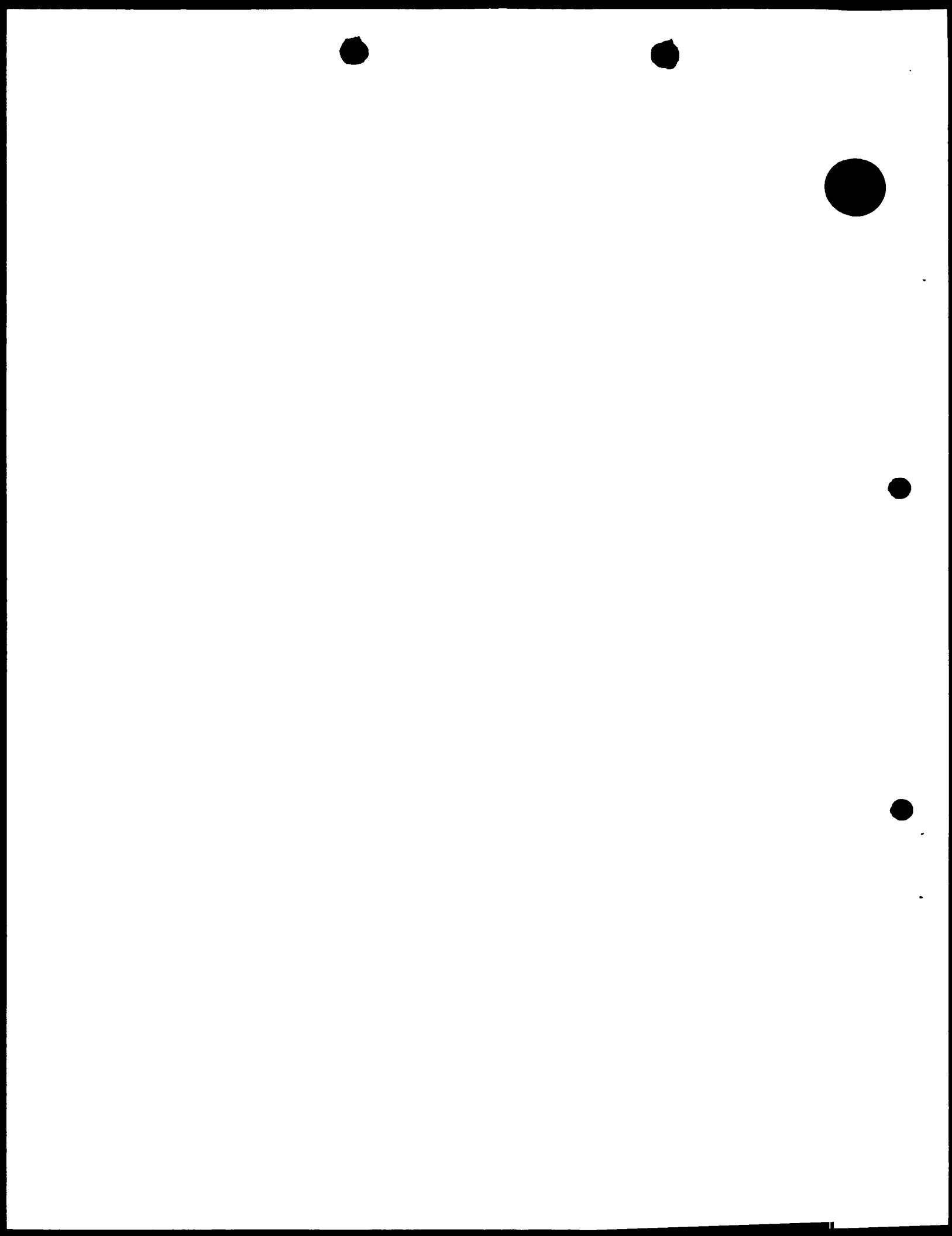
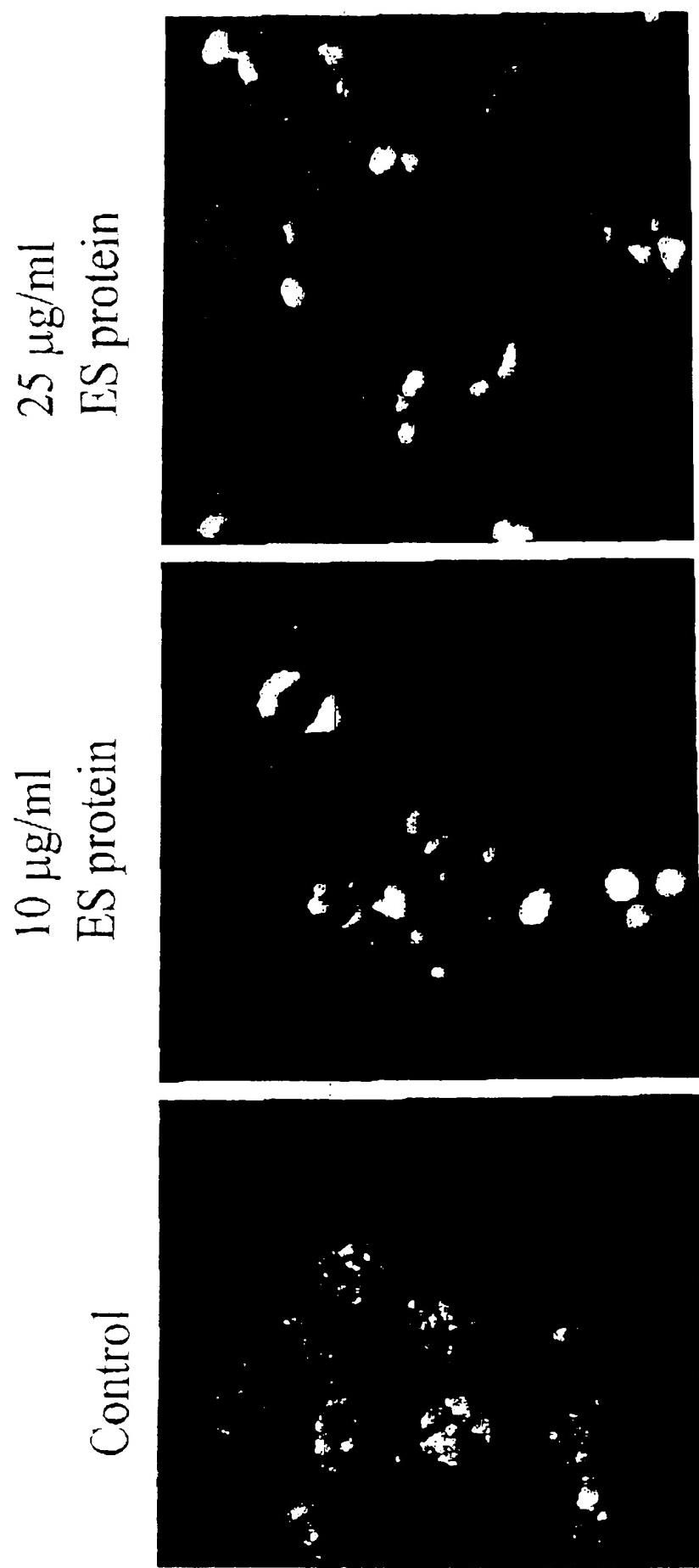


Figure 3



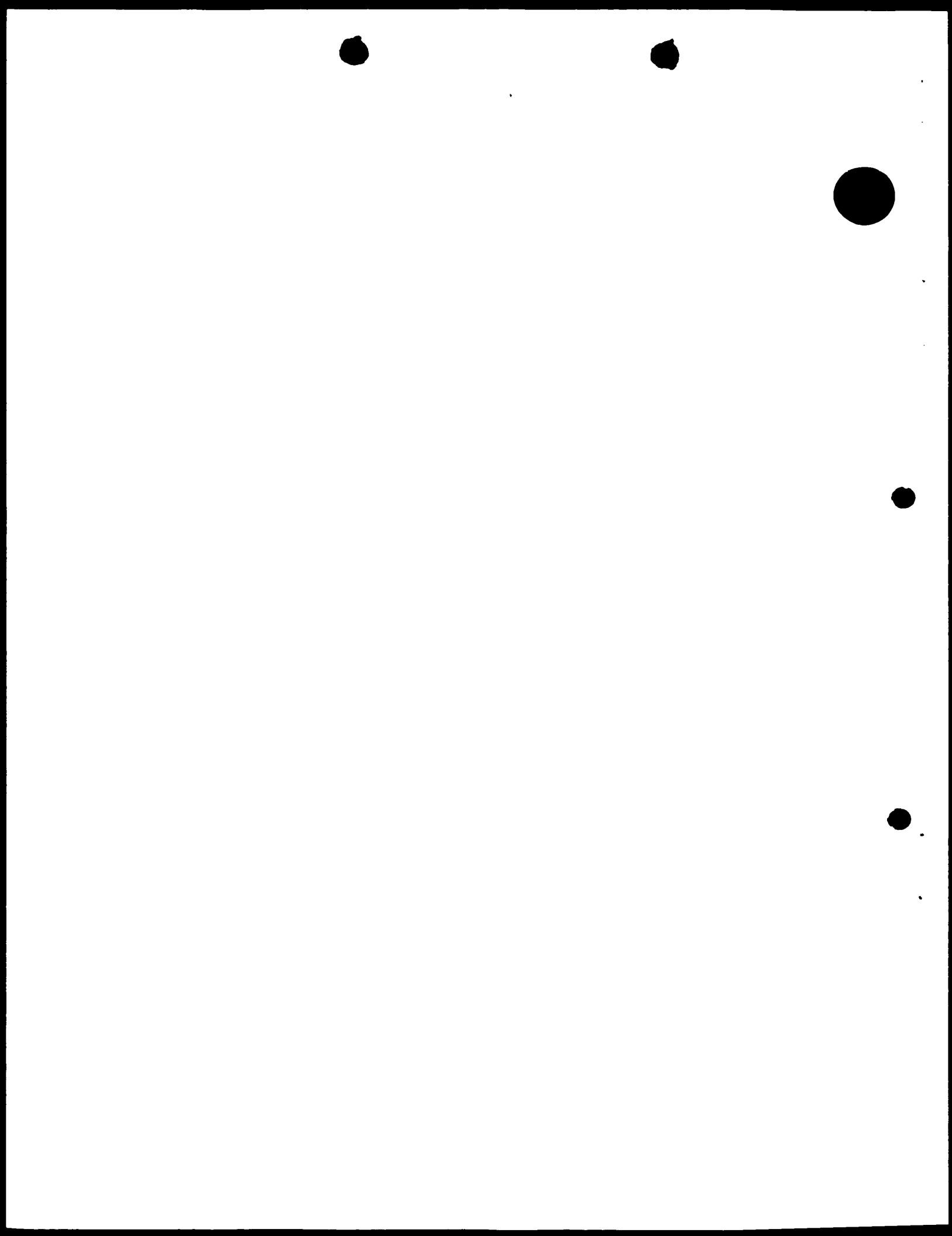
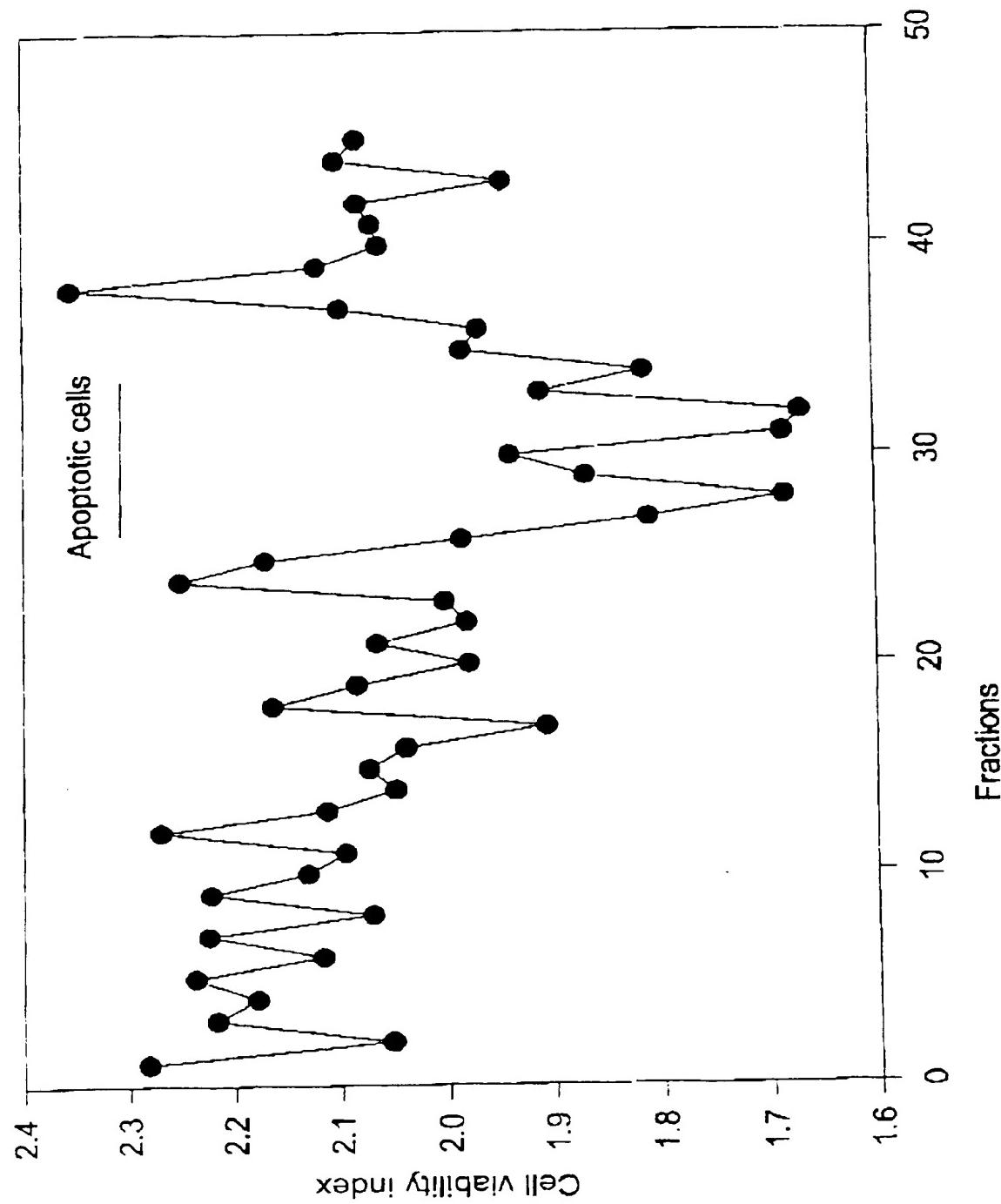


Figure 4



Experiments [unclear]

K<sub>2</sub>O<sub>2</sub> + O<sub>2</sub>

2K<sub>2</sub>O<sub>2</sub> + 2CO<sub>2</sub> → 2K<sub>2</sub>CO<sub>3</sub> + O<sub>2</sub>